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(54) Title: CONJUGATE VACCINE COMPOSED OF THE POLYSACCHARIDE MOIETY OF THE LIPOPOLYSACCHARIDE OF VIBRIO CHOLERAE 0139 BOUND TO TETANUS TOXOID

(57) Abstract: A conjugate made of the polysaccharide moiety (O-specific polysaccharide + core) of the lipopolysaccharide (LPS) of *V. cholerae* O139 (pmLPS) was prepared by derivatization of the pmLPS with adipic acid dihydrazide and coupling to tetanus toxoid (TT) by carbodiimide-mediated condensation. Said conjugate elicited high levels of IgG antibodies, peaking 3 months after the first immunization and declining slowly during the following 5 months. TT alone, or as a component of conjugate, induced mostly IgG antibodies. Antibodies elicited by the conjugate recognized both capsular polysaccharide (CP) and LPS from *V. cholerae* O139, and were vibriocidal. They were also protective in the neonatal mouse model of cholera infection. The conjugation of the O139 pmLPS, therefore, enhanced its immunogenicity and conferred T-dependent properties to this polysaccharide.

CONJUGATE VACCINE COMPOSED OF THE POLYSACCHARIDE MOIETY OF THE LIPOPOLYSACCHARIDE OF VIBRIO CHOLERAE 0139 BOUND TO TETANUS TOXOID.

Since the appearance of *Vibrio cholerae* O139 in the suburb of Madras in October 1992, epidemic cholera caused by this strain has spread rapidly throughout the Indian subcontinent (1). Clinical illness associated with *V. cholerae* O139 infection appears to be virtually identical to that due to *V. cholerae* O1 El Tor infections. However, in contrast to infection with *V. cholerae* O1, *V. cholerae* O139 infection has largely affected the adult population in *V. cholerae* O1 endemic areas, indicating a lack of protective immunity against this newly evolved strain (1). Presumably, there are differences between the immune responses against O1 and O139 strains, which may be of considerable importance in terms of protection (33). A quiescent period followed the appearance of *V. cholerae* O139 and it was thought that it was a one-time event. However, there was an upsurge of cases in Calcutta in 1996 and the O139 serogroup again became the dominant serogroup causing cholera in India by September 1996 (32). The O139 serogroup has remained present in India and Bangladesh since this last outbreak (15) and requires careful monitoring.

The epidemic and pandemic potential of *V. cholerae* O139 poses a serious threat to developing countries, and a vaccine against this novel strain is therefore required. The absence of cross-protection between *V. cholerae* O1 and *V. cholerae* O139 serogroups, documented in rabbits either immunized with live bacteria (2) or passively protected with sera of convalescent cholera patients (33), suggested that protection against cholera is LPS-specific. This is supported by the correlation observed between the protective effect of rabbit O139 antisera and anti-LPS Abs titers (25).

It has been suggested that the emergence of *V. cholerae* O139 is the result of a complex chromosomal rearrangement involving the horizontal transfer of genes encoding enzymes involved in O-specific polysaccharide (O-SP) biosynthesis (3, 8, 14, 43). Indeed, the major differences betwen *V. cholerae* O1 and *V. cholerae* O139 reside in their cell surface components. *V. cholerae* O139, unlike *V. cholerae* O1, expresses capsular polysaccharide (CP) (43, 46). Both the structure of the CP and that of the lipopolysaccharide (LPS) from *V. cholerae* O139 have been characterized

(Fig. 1) (11, 12, 28, 36). Although, O139 LPS and CP share the same repeat unit, only the CP is polymerized (12). Nevertheless, CP and LPS share common epitopes (43).

Several oral cholera vaccines, either inactivated or live attenuated, have been developed to elicit protection against this new serogroup of *V. cholerae* (10, 23, 40, 44). Various subcellular fractions of *V. cholerae* O139 administered subcutaneously have been evaluated in the rabbit ileal loop model of experimental cholera, and the immune response directed against the O139 serogroup antigen appeared to be determinant for protective immunity (4). It has been proposed that serum IgG antibodies (Abs) confer protection against enteric diseases by inactivating the inoculum on the mucosal surfaces (38). Systemic administration of IgG Abs specific for the O-SP of *V. cholerae* O1 was found to protect neonatal mice against loss of weight and death following intragastral challenge with *V. cholerae* O1 (5). A *V. cholerae* O139 CP-tetanus toxoid conjugate vaccine induced protection in the rabbit ileal loop model of experimental cholera (24). More recently, *V. cholerae* O139 CP conjugated with a recombinant mutant diphtheria toxin was shown to elicit high levels of serum anti-CP IgG in mice with vibriocidal activity (30). Other vaccines based on polysaccharide-protein conjugate to prevent cholera were developed (16, 17).

In this study, a conjugate prepared with the polysaccharide moiety (O-SP + core) of the LPS (pmLPS) from *V. cholerae* O139 bound to tetanus toxoid (TT) has been synthesized. The synthesis, characterization and immunologic properties in mice of this conjugate were assessed.

Said conjugate vaccine elicits anti-O139 Abs in mice; the immunologic properties of these Abs were also studied. As observed in many LPS from various Gram-negative bacteria, the *V. cholerae* pmLPS is attached to the lipid A portion of the molecule through Kdo (12, 48). This bond is cleaved by mild acid hydrolysis (Fig. 1) to release a polysaccharide bearing a Kdo residue at its reducing end (22). The use of the carboxylic group of the Kdo moiety for polysaccharide-protein coupling results in a saccharide with a single terminal active site for conjugation. This single-end activated pmLPS showed a high potential for use as a vaccine: (i) the O139 specific antigenic determinant(s) are conserved; (ii) it is the simplest conjugate configuration in which polysaccharide chains radiate from the

protein carriers; (iii) the coupling procedure is the easiest to control, producing well-defined non-crosslinked, water soluble conjugate molecules of known configuration (22).

It has been shown that phenol-water extraction of capsulated bacteria yields a mixture of LPS and CP in the aqueous phase (11, 28, 37, 46). To confirm the effective separation of LPS from CP after further purification steps, these two types of cell surface polysaccharide were identified by tricine SDS-PAGE using differential staining. Only the rapidly migrating material, corresponding to LPS, was silver stained, but the slowly migrating forms of the O139 antigen were not. This result is consistent with the previous observation that O139 CP is not stained with silver (34). It is thought that the silver staining of polysaccharides depends on the presence of periodate-sensitive cis-hydroxyl groups in the monosaccharide residues (9). Thus, as the O139 CP repeating unit, unlike the LPS core, lacks cis-hydroxyls (11, 12, 28, 36) it is not silver stained. However, this CP, which is acidic, is stained by the cationic dye, Alcian Blue.

The various aspects of the present invention are based upon the discovery of the properties of the polysaccharide moiety (O-SP+core), also named pmLPS, of the LPS from *V. cholerae* 0139 and more specially of a conjugate prepared with this polysaccharide moiety bound to tetanus toxoid (TT).

Therefore, the invention provides an immunogenic composition against *Vibrio* infection comprising an O-SP unit of LPS of *Vibrio* associated to a core molecule of LPS of *Vibrio* or a polymer of said composition.

As used herein, a "polymer" of the composition of the invention means a composition comprising several, at least two, O-SP+core (pmLPS) linked by any means one to another or together.

According to a preferred embodiment of said immunogenic composition, the O-SP unit associated to the core molecule of LPS of *Vibrio* is part of a conjugate further comprising a carrier protein.

Carrier proteins are known from the one skilled in the art. Examples of bacterial carrier proteins are diphteria toxins, tetanus toxoïd...

Preferably:

- the *Vibrio* O-SP unit and core molecule are bound to the carrier protein of the conjugate by a covalent link.

- the carrier protein is a bacterial protein, for instance tetanus toxoid.

According to another preferred embodiment of said immunogenic composition, it further comprises an adjuvant and/or a pharmaceutically acceptable carrier.

Adjuvants and pharmaceutically acceptable carriers are known from the one skilled in the art.

Examples of species from Vibrionacae family are : *V. alginolyticus, V. cholerae, V. cincinnatiensis, V. diabolicus, V. diazotrophicus, V. harveyi, V. logei, V. natriegens, V. nereis, V. splendidus, V. tubiashii, V. halioticoli, V. ichthyoenteri, V. pectenicida* and *V. wodanis*.

According to yet another preferred embodiment of said immunogenic composition, the LPS is from *Vibrio* cholera, more preferably from *Vibrio* cholera serogroup O139.

According to yet another preferred embodiment of said immunogenic composition, the O-SP unit and the core are from two different *Vibrio*.

The present invention also includes a vaccine composition comprising an O-SP unit of LPS of *Vibrio* associated to a core molecule of LPS of *Vibrio* or a polymer of said composition, said vaccine composition being protective against infection from *Vibrio*.

According to a preferred embodiment of said vaccine composition, it is protective against infection from *Vibrio cholerae*, preferably against infection from *Vibrio cholerae* serogroup O139.

The invention further includes a method for preparing a conjugate as defined above, i.e. comprising an O-SP unit associated to the core molecule of LPS of Vibrio and a carrier protein, comprising:

a) providing LPS from *Vibrio*;

b) hydrolyzing the lipid A-core linkage for obtaining an O-SP unit associated to a core molecule;

c) derivatizing the O-SP unit associated to the core molecule of step b);

d) bounding the derivatized the O-SP unit associated to the core molecule of step c) to a carrier protein;

e) collecting the O-SP unit associated to the core molecule bound to the carrier protein in step d).

According to said method, the O-SP unit associated to the core molecule is bound to the carrier protein by a covalent link.

More preferably, in said method:

- the carrier protein is a bacterial protein.
- the bacterial protein is tetanus toxoid.
- LPS of step a) is from *Vibrio cholerae*, more preferably from *Vibrio cholerae* serogroup O139.

The derivatization ratio of pmLPS, an essential step in the coupling procedure of the invention, was lower than usual with other polysaccharides (13, 16). Nevertheless, the polysaccharide/protein ratio (0.99 mol/mol) obtained herein was sufficient for a strong IgG response in immunized mice. The unconjugated pm-LPS elicited mostly IgM Abs, whereas only low levels of IgG anti-LPS Abs were detected. This response was similar to those previously reported for polysaccharides tested in mice (45). In contrast, the pmLPS-TT conjugate elicited mostly IgG anti-LPS Abs, which were boosted following reimmunization. Moreover, after the fourth immunization, a high level of these IgG Abs was maintained for 5 months. It was found that pm-LPS-TT had typical T-dependent properties. Similar results have been obtained with O-SP from several other enteric bacterial pathogens (7, 29).

Interestingly, Abs obtained in mice immunized with pmLPS conjugated to TT recognized both O-SP and CP purified from *V. cholerae* O139. This result is entirely consistent with the observation that CP and LPS share common epitope(s) expressed by a common hexasaccharide unit (12, 43). This cross-reactivity between O139 pmLPS and CP accounts for the finding of the invention that pmLPS-TT Abs reacted with both encapsulated and non-encapsulated *V. cholerae* O139 strains, and is consistent with observations that protection against *V. cholerae* O139 can be mediated by Abs directed against either the LPS or CP of this novel

cholera vibrio (24, 25, 33, 34, 39). The invention demonstrates the efficiency of a conjugated pmLPS in eliciting an IgG response in mice and justifies clinical evaluation of this *V. cholerae* O139 conjugate.

The invention also includes a method for immunizing human or animal against *Vibrio* infection, wherein said method comprises administration to said human or animal of a composition as defined hereabove, wherein *Vibrio* infection is preferably an infection from *Vibrio cholerae* and more preferably from *Vibrio cholerae* serogroup O139.

Therefore, the invention also includes the use of a composition comprising a conjugate compound comprising an O-SP unit of LPS of *Vibrio* associated to a core molecule of LPS of *Vibrio* bound to a protein carrier for the preparation of a medicament for preventing a *Vibrio* infection, more preferably a *Vibrio* infection from *Vibrio cholerae* and more preferably from *Vibrio cholerae* serogroup O139.

The instant invention further includes a conjugate compound comprising an O-SP unit of LPS of *Vibrio* associated to a core molecule of LPS of *Vibrio* bound to a protein carrier.

According to a preferred embodiment of said conjugate, the *Vibrio* O-SP unit associated to the *Vibrio* core molecule is bound to the protein carrier by a covalent link.

According to another preferred embodiment of said conjugate, the protein carrier is a bacterial protein, more preferably tetanus toxoid.

According to yet another embodiment of said conjugate, the *Vibrio* LPS is from *Vibrio cholerae*, more preferably from *Vibrio cholerae* serogroup O139.

According to yet another embodiment of said conjugate, the O-SP unit and the core are from two different *Vibrio*.

The present invention will be further illustrated by the additional description which follows, which refers to examples of preparation and use of the conjugate according to the invention.

It should be clearly understood however that these examples are given solely as illustration of the subject of the invention and do not constitute in any manner a limitation thereof.

Description of the drawings

- FIG. 1. Overall structure of the LPS of *V. cholerae* O139. The O-specific polysaccharide (O-SP) and the core structure are taken from Cox *et al.* (11, 12) and the lipid A structure is arranged according to Kabir (26) and Wilkinson (48). The arrow indicates the lipid A-core bond hydrolyzed by acetic acid treatment: this treatment releases the polysaccharide moiety (O-SP + core) of the LPS (pmLPS).

- FIG. 2. Analysis of polysaccharide preparations of *V. cholerae* O139. (A) Tricine SDS-PAGE (16.5%). The gel was stained with silver. (B) SDS-PAGE (10%). The gel was pretreated with Alcian Blue, a cationic dye that binds acidic polysaccharides, prior to silver staining. (C) Immunoblot analysis with hyperimmune O139 mice antiserum as the probe. M_r values are shown on the left. MF: migration front.

- FIG. 3. Double immunodiffusions. A, mAb anti-LPS O139; 1, pmLPS O139; 2, LPS O139; 3, CP O139; 4, LPS O1; 5, derivatized pmLPS O139; 6, pmLPS-TT.

- FIG. 4. Time course of amounts of IgM (■) and IgG (●) anti-O139 Abs, and O139 vibriocidal Abs titer (▲) in serum of a single mouse immunized four times (arrows) with pmLPS-TT.

- FIG 5. Protective activity of anti-pmLPS-TT Abs against challenge with 10 x LD_{50} of *V. cholerae* O139 in the suckling-mouse model: NI, pooled non immune sera; IS, pooled immune sera obtained on days 152 and 231 from mice immunized with pmLPS-TT. Health status was scored 48 h after challenge.

<u>EXAMPLE 1 :</u> Preparation and characterization of LPS, pmLPS, and CP.

V. cholerae O139 (strain MO45, kindly provided by Y. Takeda, Kyoto University, Japan) was grown in Tryptic Soy agar (Difco) at 37°C for 18 h. LPS was obtained by hot phenol water extraction (47), followed by enzymatic treatment (DNase, RNase and protease) and ultracentrifugation. The pellet, containing the LPS had 0.5% (w/v) protein and less than 0.2% (w/v) nucleic acid. LPS was treated with acetic acid to hydrolyze the lipid A-core linkage (Fig. 1) (19). The resulting product is referred to as pmLPS. For the preparation of CP, LPS was removed from the ultracentrifugation supernatant by passage through a Sephacryl S-200 column in a buffer containing deoxycholic acid (37). Void volume fractions

containing CP, detected by refractive index and 10% SDS-PAGE in gels treated with Alcian Blue (a cationic dye that binds acidic polysaccharides) prior to silver staining (9), were dialyzed extensively against 10% (v/v) ethanol to remove deoxycholic acid (37). The LPS had 2 x 10^4 endotoxin units/μg and the pmLPS had 10 endotoxin units/μg as assessed by the *Limulus* amebocyte lysate assay (21). This reduction by a factor of 2000 is consistent with previous data (16, 42). LPS from *V. cholerae* O139 gave two dense silver-stained bands (41) in 16.5% tricine SDS-PAGE (31) with M_r values of approximately 4,000 and 6,200 (Fig. 2A). LPS from *V. cholerae* O1 gave two bands with M_r values of 4,000 and 15,000 (Fig. 2A). This is consistent with the observation that O139 O-SP has one hexasaccharide unit (12) whereas O1 O-SP has 12-18 repeating monosaccharide units (27). In 10% SDS-PAGE (Fig. 2B), in gels treated with Alcian Blue prior to silver staining, O139 LPS gave one band with a smear at the bottom of the gel and O139 CP gave two bands with M_r values of 100,000 and 200,000, consistent with the polymerized structure of this polysaccharide (28, 36). Both *V. cholerae* O139 LPS and CP were recognized by an anti-O139 hyperimmune mice serum in immunoblotting experiments (Fig. 2C). This is consistent with the observation that O139 O-SP shares an epitope with O139 CP (43). This hyperimmune mouse serum did not react with *V. cholerae* O1 LPS in the same conditions. Monoclonal Ab (mAb), prepared as previously described (6), were screened by ELISA against purified O139 LPS and checked for specificity by immunoblot analysis against O139 and O1 LPS, and by agglutination with *V. cholerae* O139 and O1 bacterial cells. Clone B-16-5, IgM class, was selected for its high avidity to O139 pmLPS and O139 CP, as determined by ELISA inhibition. Double immunodiffusion assay showed a single band of precipitate between LPS, pmLPS, CP, and the B-16-5 mAb (Fig. 3). That pmLPS yielded a line of identity with LPS suggests that the O-139-specific antigenic determinant was preserved during the purification of the pmLPS. No cross-reactivity was observed with LPS from *V. cholerae* O1, serotype Inaba. The ^1H and ^{31}P NMR spectra of the pmLPS, recorded on a Bruker AC 300P spectrometer, were identical to those previously reported (11). The ^1H NMR spectrum confirmed the absence of small organic molecules.

EXAMPLE 2: **Preparation and characterization of *Vibrio cholerae* 0139 conjugate.**

- Bacterial strain.

V. cholerae O139, strain MO45, isolated in 1992 from a patient in Madras (India), was kindly provided by Y. Takeda (Kyoto University, Japan). This strain was used for preparation of O139 antigens.

- Preparation of LPS and pmLPS.

Bacteria were grown in Tryptic Soy Agar (Difco, Detroit, Michigan) in Roux flasks at 37°C for 18 h. Cells were resuspended in distilled water and LPS was obtained by hot phenol water extraction (47), followed by enzymatic treatment (DNase, RNase and protease) and ultracentrifugation (100,000 g for 3 h). The supernatant of ultracentrifugation was stored at –20°C. The pellet, containing the LPS, was dialyzed against distilled water and freeze-dried. This preparation contained 0.5% (w/v) protein and less than 0.2% (w/v) nucleic acids. LPS was treated with acetic acid to hydrolyse the lipid A-core linkage (Fig. 1) (19). LPS (10 mg/ml in 1% (v/v) aqueous acetic acid) was heated at 100°C for 60 min. Precipitated lipid A was removed by low-speed centrifugation (350 *g* for 10 min). The supernatant was extracted with equal volume of chloroform-ethanol (2:1). The reaction mixture was shaken vigorously and centrifuged at 10,000 *g* for 30 min. The aqueous phase was dialyzed against distilled water to remove ethanol and then freeze-dried. The resulting product is referred to as pmLPS.

Derivatization and conjugation of pmLPS.

pmLPS was derivatized with adipic acid dihydrazide (ADH) as described for *Haemophilus influenzae* b and *Shigella dysenteriae* polysaccharides (7, 22, 29). Polysaccharide (5 mg/ml in 0.2 M NaCl) was brought to pH 10.75 with 0.1 M NaOH, and an equal amount of cyanogen bromide (10 mg/ml in acetonitrile) was added. The mixture was incubated for 6 min on ice and the pH was maintained at 10.75 with 0.1 M NaOH in a pHStat 719S (Metrohm, Herisau, Switzerland). An equal volume of 0.8 M ADH in 0.5 M $NaHCO_3$ was added and the pH was adjusted to 8.5 with 0.1 M HCl for 3.5 hours at 4°C with the pHStat. Then, the reaction mixture was stirred overnight at 4°C and dialyzed against demineralized water at the same temperature for 3 days. The contents of dialysis bag were freeze-dried, reconstituted

in ultrapure water, passed through a P-10 Sephadex column and the void volume fractions were pooled and freeze-dried.

The derivatized pmLPS (pmLPS-AH) was dissolved in 0.2 M NaCl at 5 mg/ml. An equal weight of TT (Pasteur-Mérieux, Marcy-l'Etoile, France) was added and the pH was adjusted at 5.3 with 0.1M HCl. 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC) was added to a final concentration of 0.05 M and the pH was maintained with the pHStat for 4 hours at 4°C. The reaction mixture was dialyzed against PBS at 4°C for 2 days and then passed through a column (1.5 by 90 cm) of CL-6B Sepharose in PBS. TT was detected by measuring the optical density at 280 nm and polysaccharide by determining the refractive index.

The extent of derivatization of the activated pmLPS was calculated as the ratio ADH/polysaccharide and was 5.2% (mol/mol). For the conjugate, the pmLPS/protein (wt/wt) ratio was 1.90%, corresponding to a 0.99 mol/mol ratio. The yield was 9.6%, as calculated by the ratio of the amount of the saccharide in the conjugate over the initial amount of derivatized polysaccharide. In double-immunodiffusion assays, mAb B-16-5 gave a line of identity with pmLPS, derivatized pmLPS and TT-pmLPS, suggesting that the O139 antigenic determinant common to O-SP and CP was preserved during the conjugation of the pmLPS (Fig. 3).

Characterization of polysaccharide preparations.

Double immunodiffusion was performed in 1% (w/v) agarose (Indubiose® IBF, Villeneuve-la-Garenne, France) in NaCl 0.5 M with 0.05% (w/v) NaN_3. Protein concentration was determined by Lowry's assay, using bovine serum albumin as the standard. The residual LPS, assayed by the *Limulus* amoebocyte Lysate (LAL) assay (Bio-Whittaker, Walkersville, Md) is expressed in endotoxin units relative to the US standard (21). Nucleic acids were detected electrophoretically by using 1% agarose plate with λ DNA hydrolyzed by *Hind*III as the standard. LPS, pmLPS and CP were analysed by 10% SDS-PAGE and either stained with 0.5% (w/v) Alcian Blue (9) prior to silver staining (41) or electrotransferred to nitrocellulose for immunoblot analysis. LPS and pmLPS were analyzed by Tricine SDS-PAGE (31, 49) using a 16.5% (w/v) running gel and a 4% stacking gel and silver stained. The ^{1}H and ^{31}P NMR spectra of the pmLPS were recorded on a Bruker AC 300P spectrometer.

EXAMPLE 3 : PREPARATION OF *Vibrio cholerae* O1 CONJUGATE

- Bacterial strain.

V. cholerae O1 CNRVC 950707, serotype Inaba strain isolated in 1995 from a patient in Mali, was used for preparation of O1 LPS.

- Preparation of LPS and pmLPS.

Same methods as described for *V. cholerae* O139 (see example 2)

Derivatization and conjugation of pmLPS.

The only modification concerned the temperature of the incubation steps of the reagents in the pHStat which was room temperature for O1 conjugate instead of +4°C as previously described for O139 conjugate (see example 2).

EXAMPLE 4 : Anti-O139 and anti-TT Abs response of mice.

- Immunization

Six week old female BALB/c were injected subcutaneously with 2.5 μg of pmLPS O139 alone, or as a conjugate (see example 2), as described in the footnote of Table I. A group of mice was immunized similarly with 2.5 μg of TT. LPS and TT Ab levels were determined by ELISA. Plates were coated with either LPS or TT. Serial twofold dilutions of mouse sera (1/100 to 1/6,400) were analyzed. The secondary Abs used were either peroxidase-conjugated anti-mouse IgG (γ chain-specific), or IgM (μ chain-specific). The results were calculated for each immunoglobulin class, as a percent of a high-titered reference serum arbitrarily assigned a value of 100 ELISA units (EU) by parallel line analysis with a program from the Centers for Disease Control and expressed as the geometric mean (35). Following the same method, anti-TT Ab level was expressed with respect to an hyperimmune mouse pooled standard serum prepared by repeated immunizations of mice with TT. Serum anti-O139 Ab titers are shown in Table I. Pre-immune sera and PBS control sera contained no detectable levels of Abs. After the second immunization, pmLPS elicited a moderate IgM response and a very weak IgG response, consistent with the response induced by a T-independent antigen. After the third immunization with pmLPS-TT, IgM titers were equivalent to those elicited in response to pmLPS. After the fourth immunization, pmLPS-TT elicited a very much higher IgG response than pmLPS ($P = 0.0011$), lasting at least 231 days ($P = 0.0046$).

This IgG response demonstrates a booster effect and an immunoglobulin isotype switch. This strongly suggests that the pmLPS was functionally converted, due to the protein carrier effect, into a T-dependent antigen. In inhibition ELISA (42), the binding of anti-pmLPS-TT antibodies to O139 LPS was inhibited by either O139 LPS (amount of antigen yielding 50% inhibition: 8 μg/ml) or O139 CP (1 μg/ml). Serum anti-TT Ab titers are shown in Table I. Pre-immune sera contained no detectable levels of anti-TT Abs. After the third immunization, pmLPS-TT elicited a significant increase in anti-TT IgG levels ($P < 0.01$), similar to that in mice immunized with TT alone.

- Vibriocidal Abs response.

The vibriocidal tests were performed as previously described (5) with two-fold dilutions (beginning with an initial 1:10 dilution) using *V. cholerae* O139 strain MO10-T4, a spontaneous nonencapsulated variant of MO10 (43), kindly provided by A. Weintraub (Karolinska Institute, Huddinge, Sweden), as the target strain and Guinea pig serum as the source of complement. The vibriocidal titer was defined as the reciprocal of the highest dilution of serum causing 100% bacterial lysis. Controls for each assay included, in addition to the usual cell control and complement control, a positive hyperimmune control serum with a titer of 1/2560. Consecutive sera of one mouse immunized with pmLPS-TT were tested for vibriocidal activity (Fig. 4). There was a correlation between the kinetics of the vibriocidal Abs titer and the anti-O139 IgG level (correlation coeficient = 0.89). Findings for sera from other mice immunized with pmLPS-TT supported this correlation.

- Protective activity of anti-pmLPS-TT Abs.

Suckling Swiss mice 5 days old and weighing 3.3 to 4.4 g were used for oral challenge experiments with *V. cholerae* O139. A *V. cholerae* O139 strain, isolated in 1992 from a patient in India and selected for its capacity to produce high levels of cholera toxin (5 μg/ml), was used for oral challenge in mice. After removing secreted cholera toxin, a dose of 3.5×10^8 *V. cholerae* cells (10 times the 50% lethal dose), preincubated for 30 min at 37°C with immune serum at various dilutions in 0.1 ml, was delivered into the stomach with a blunt-tip feeding needle. Groups of mice that received vibrio suspension alone, PBS alone or *Vibrio* suspension with non-immunized mice serum served as controls. Mice were maintained at 30°C for 48 h or

until death, and all surviving mice were scored as well or ill at 48 h. Mice were considered ill if they met all of the following criteria: diarrhea, markedly reduced skin turgor and poor response to stimuli. Mice that received pooled immune sera, collected on days 152 and 231 from mice immunized with pmLPS-TT, diluted 1:5, were significantly protected (Fig. 5). The level of protection decreased as the dilution of the pooled immune sera increased: protection was therefore dependent on dose. No protection was observed in mice that received pooled non-immune control sera.

TABLE I. Geometric mean ELISA titer (25th-75th percentiles) of serum anti-LPS and anti-TT Abs elicited in mice following immunization with pmLPS alone or as a conjugate[a]

Day[b]	Abs and immunogen					P[c]
	Anti-TT IgG	Anti-LPS IgM		Anti-LPS IgG		
	pmLPS-TT	pm-LPS	pmLPS-TT	pm-LPS	pmLPS-TT	
0*	<1	<1	<1	<1	<1	NS[d]
7	<1	2.8 (2.3-3.5)	1.8 (1.5-2.2)	1.4 (1.3-1.5)	1.2 (0.9-1.6)	NS
14*						
21	9.3 (5.3-12.2)	7.9 (4.8-14.2)	6.2 (5.4-8.8)	<1	1 (0.8-1)	NS
28*						
35	61.9 (52.8-109.5)	17.5 (7.5-41.8)	17.7 (12.3-24.3)	<1	1.8 (0.9-4.6)	NS
56*						
63	193.5 (155.9-219.2)	6.5 (3.6-11.4)	8.1 (6.2-9)	2 (1.2-4.9)	11.7 (8.1-15.3)	0.0011
91	280.5 (204.1-454.8)	4.2 (3.1-6.8)	7.5 (5.7-8.8)	<1	36.3 (11.7-67.3)	0.0357
119	191.3 (156.4-308.8)	4.4 (2.5-6.9)	2.8 (2-3.5)	1.2 (1.1-3.3)	21.4 (7.3-45.8)	0.0249
152	209.5 (181.1-295.3)	4.2 (2.9-6.4)	12.7 (10.1-15.3)	2.5 (2.1-3.1)	29.9 (14.8-78.3)	0.0131
190	192.2 (147.9-277.9)	4.6 (3.5-6.6)	7.2 (6-9.5)	1.3 (1.1-2.2)	30.6 (15.7-72.3)	0.0055
231	183[e] (144.4-249.5)	4.5 (3.1-6.5)	6.3[e] (4.5-9.3)	<1	23.8[e] (12.3-60.6)	0.0046

[a] Ten mice were injected subcutaneously with saline solutions containing 2.5 μg of the antigen three times at 2 weeks intervals and were then given a fourth injection 4 weeks later. The mice were bled 7 days after each injection and then again each month for 6 months after the fourth injection.
[b] Days of immunization are marked with an asterisk.
[c] Comparison of titers of anti-LPS IgG elicited by pmLPS-TT versus pmLPS (P values are calculated by Student's t test).
[d] NS, not significant.
[e] Nine mice were tested.

EXAMPLE 5 : **Results obtained with the *V. cholerae* O1 conjugate**

Mice were immunized as previously described for *V. cholerae* O139 conjugate. Serum anti-O1 antibodies titer at days 7 and 68 after the first immunization are shown in Table II.

Table II. ELISA titers of serum anti-*V. cholerae* O1 LPS antibodies in mice 7 days and 68 days after immunization with *V. cholerae* O1 conjugate[a]

	ELISA titer			
Mice Number	Anti-LPS IgM		Anti-LPS IgG	
	Day 7 after immunization	Day 68 after Immunization	Day 7 after immunization	Day 68 after immunization
1	< 1	4	2	6
2	< 1	5	1	82
3	< 1	6	2	13
4	< 1	6	4	18
5	< 1	7	2	13
6	< 1	4	2	7
7	< 1	3	2	18
8	< 1	13	2	36
9	< 1	4	2	14
10	< 1	9	3	7

[a] Mice were injected subcutaneously with saline solution containing 2.5 µg of the conjugate three times at 2-weeks interval and were given a fourth injection 4 weeks later. The mice were bleed at days 7 and 68 after the first immunization.

The *V. cholerae* O1 conjugate elicited high levels of IgG antibodies compared to low levels of IgM. Conjugation of the *V. cholerae* O1 polysaccharide, therefore, conferred T-dependent properties on this polysaccharide.

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CLAIMS:

1. An immunogenic composition against *Vibrio* infection comprising an O-SP unit of LPS of *Vibrio* associated to a core molecule of LPS of *Vibrio* or a polymer of said composition.

2. The immunogenic composition of claim 1, wherein the O-SP unit associated to the core molecule of LPS of *Vibrio* is part of a conjugate further comprising a carrier protein.

3. The immunogenic composition of claim 2, wherein the *Vibrio* O-SP unit and core molecule are bound to the carrier protein of the conjugate by a covalent link.

4. The immunogenic composition of claim 2, wherein the carrier protein is a bacterial protein.

5. The immunogenic composition of claim 4, wherein the bacterial protein is tetanus toxoid.

6. The immunogenic composition of claim 1, wherein said composition further comprises an adjuvant and/or a pharmaceutically acceptable carrier.

7. The immunogenic composition of claim 1, wherein the LPS is from *Vibrio* cholera.

8. The immunogenic composition of claim 1, wherein the LPS is from *Vibrio* cholera serogroup O139.

9. A vaccine composition protective against infection from *Vibrio* wherein said vaccine composition comprises an immunogenic composition according to claim 1.

10. The vaccine composition of claim 9, wherein said vaccine composition is protective against infection from *Vibrio cholerae*.

11. The vaccine composition of claim 10, wherein said vaccine composition is protective against infection from *Vibrio cholerae* serogroup O139.

12. A method for preparing a conjugate comprising an O-SP unit of LPS from a *Vibrio* associated to a core molecule of LPS of a *Vibrio* bound to a protein carrier, said method comprising:

a) providing LPS from a *Vibrio*;

b) hydrolyzing the lipid A-core linkage for obtaining an O-SP unit associated to a core molecule;

c) derivatizing the O-SP unit associated to the core molecule of step b);

d) bounding the derivatized the O-SP unit associated to the core molecule of step c) to a carrier protein;

e) collecting the O-SP unit associated to the core molecule bound to the carrier protein in step d.

13. The method of claim 12, wherein the O-SP unit associated to the core molecule are bound to the carrier protein by a covalent link.

14. The method of claim 12, wherein the carrier protein is a bacterial protein.

15. The method of claim 14, wherein the bacterial protein is tetanus toxoid.

16. The method of claim 12, wherein LPS of step a) is from *Vibrio cholerae.*

17. The method of claim 16, wherein LPS of step a) is from *Vibrio cholerae* serogroup O139.

18. Use of a composition comprising a conjugate compound comprising an O-SP unit of LPS of *Vibrio* associated to a core of LPS of *Vibrio* bound to a protein carrier for the preparation of a medicament for preventing a *Vibrio* infection.

19. Use of claim 18, wherein *Vibrio* infection is an infection from *Vibrio cholerae.*

20. Use of claim 19, wherein *Vibrio cholerae* infection is an infection from *Vibrio cholerae* serogroup O139.

21. A conjugate compound comprising an O-SP unit of LPS of *Vibrio* associated to a core molecule of LPS of *Vibrio* bound to a protein carrier.

22. The conjugate compound of claim 21, wherein the *Vibrio* O-SP unit associated to the *Vibrio* core molecule is bound to the protein carrier by a covalent link.

23. The conjugate compound of claim 21, wherein the protein carrier is a bacterial toxin.

24. The conjugate compound of claim 23, wherein the bacterial toxin is tetanus toxoid.

25. The conjugate compound of claim 21, wherein the *Vibrio* LPS is from *Vibrio cholerae*.

26. The conjugate compound of claim 25, wherein the *Vibrio cholerae* LPS is from *Vibrio cholerae* serogroup O139.

27.The composition of claim 1 wherein the O-SP unit and the core are from two different *Vibrio*.

28.The conjugate of claim 21, wherein the O-SP unit and the core are from two different *Vibrio*.

29. A method for immunizing human or animal against *Vibrio* infection, wherein said method comprises administration to said human or animal of a composition as defined hereabove, wherein *Vibrio* infection is preferably an infection from *Vibrio cholerae* and more preferably from *Vibrio cholerae* serogroup O139.

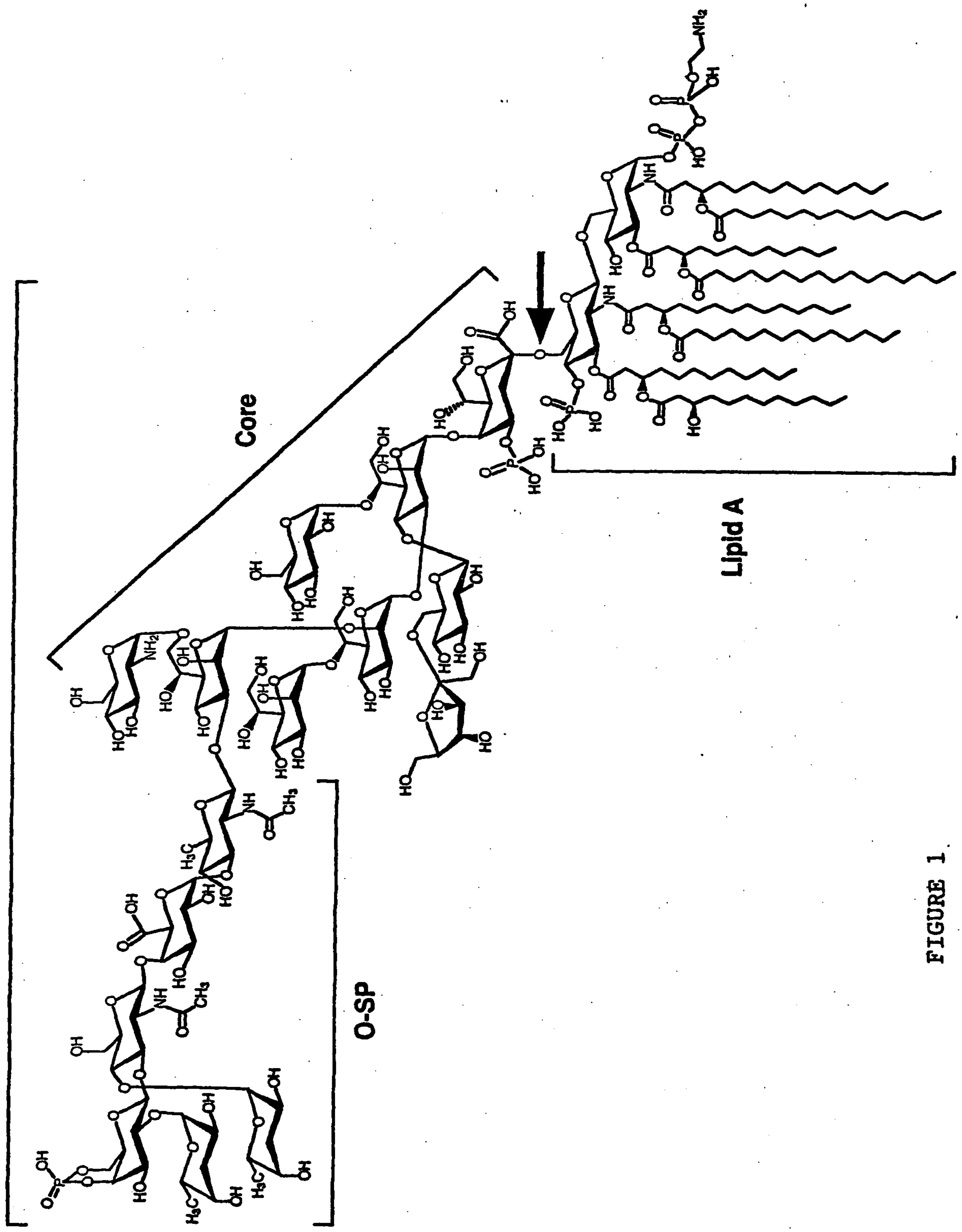

FIGURE 1

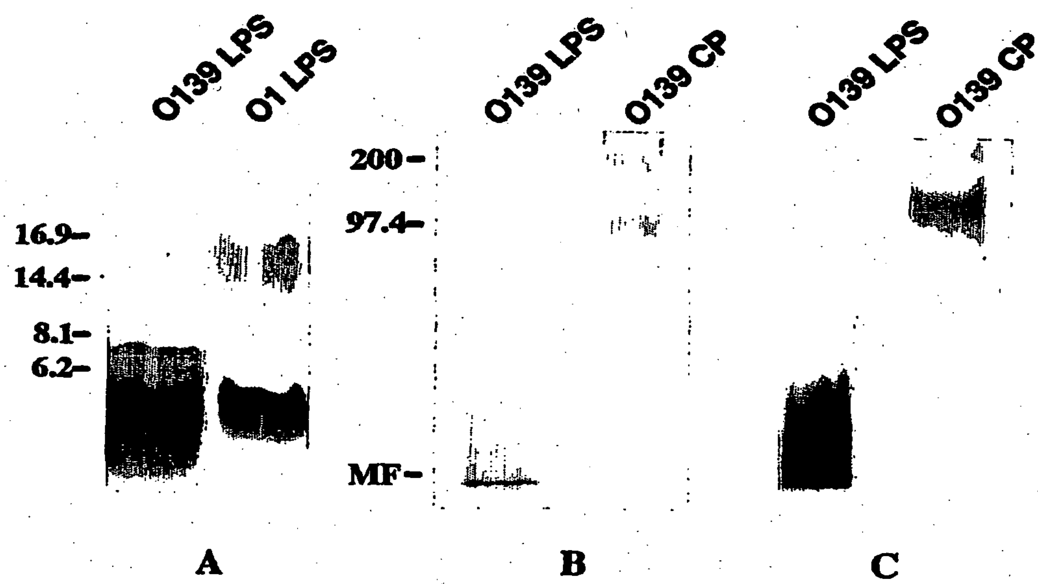
O139 LPS
O1 LPS
16.9
14.4
8.1
6.2
O139 LPS
O139 CP
200
97.4
MF
O139 LPS
O139 CP
A
B
C

FIGURE 2

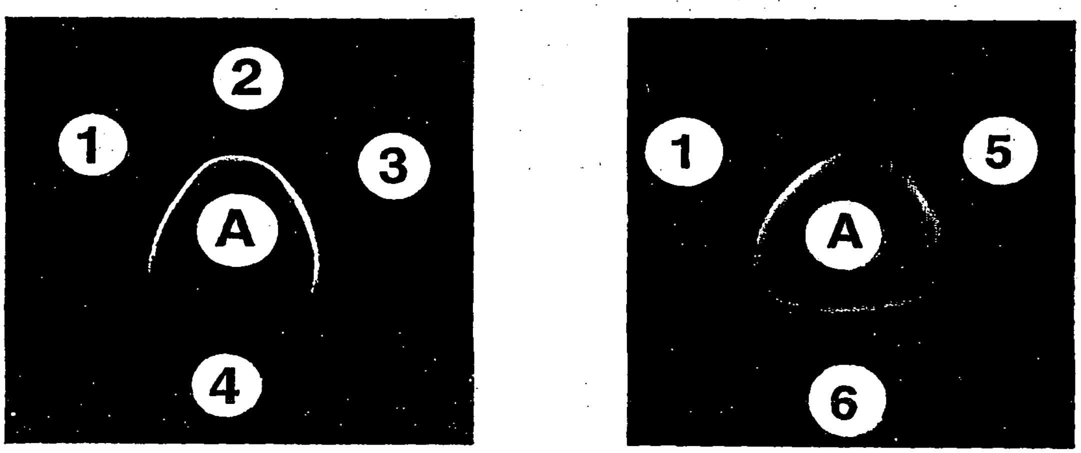

FIGURE 3

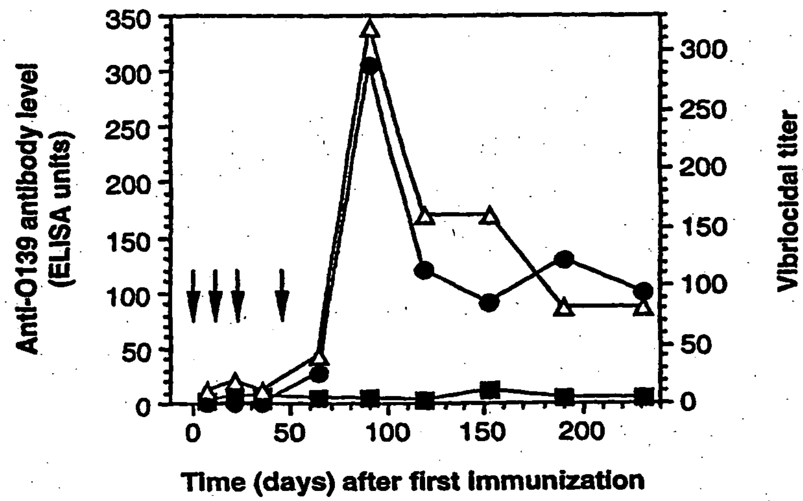
350
300
250
200
150
100
50
0
Anti-O139 antibody level (ELISA units)
300
250
200
150
100
50
0
Vibriocidal titer
0
50
100
150
200
Time (days) after first immunization

FIGURE 4

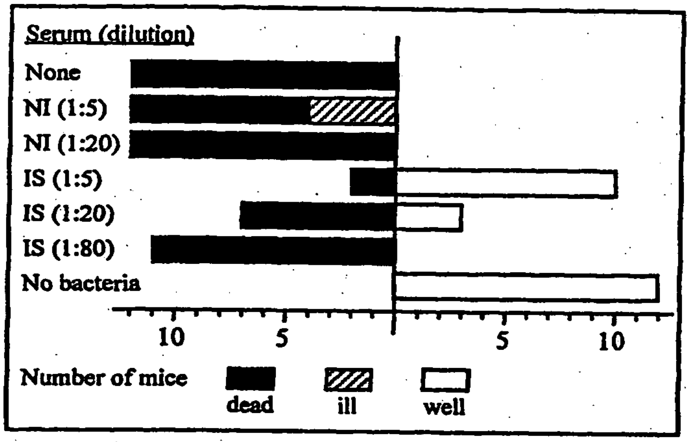
Serum (dilution)
None
NI (1:5)
NI (1:20)
IS (1:5)
IS (1:20)
IS (1:80)
No bacteria
10
5
5
10
Number of mice
dead
ill
well

FIGURE 5

INTERNATIONAL SEARCH REPORT

International Application No
PCT/IB 02/02184

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K39/106 A61P31/04 A61K39/385

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, WPI Data, PAJ, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 93 13797 A (US HEALTH) 22 July 1993 (1993-07-22)	1-7, 9-16, 18-25, 27-29
Y	page 1, line 30-page 2, line 8; page 2, line 34-page 3, line 3; page 4, lines 9-28; page 5, lines 17-34; page 6, lines 3-21; page 15, lines 7-9; examples 1-4	8,17,26
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 August 2002	12/09/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Renggli, J

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	JONSON GUNHILD ET AL: "Immune mechanisms and protective antigens of Vibrio cholerae serogroup 0139 as a basis for vaccine development." INFECTION AND IMMUNITY, vol. 64, no. 9, 1996, pages 3778-3785, XP002209046 ISSN: 0019-9567 abstract	8,17,26
X	GUPTA RAJESH K ET AL: "Phase 1 evaluation of Vibrio cholerae 01, serotype inaba, polysaccharide-cholera toxin conjugates in adult volunteers." INFECTION AND IMMUNITY, vol. 66, no. 7, July 1998 (1998-07), pages 3095-3099, XP002209047 ISSN: 0019-9567 p. 3096 "Polysaccharide" and "Conjugation"; abstract	1-4,6,7, 9-14,16, 18-23, 25,29
X	JERTBORN M ET AL: "Intestinal and systemic immune responses in humans after oral immunization with a bivalent B subunit-01/0139 whole cell cholera vaccine" VACCINE, BUTTERWORTH SCIENTIFIC. GUILDFORD, GB, vol. 14, no. 15, 1 October 1996 (1996-10-01), pages 1459-1465, XP004070959 ISSN: 0264-410X page 1460, left-hand column -page 1460, right-hand column page 1464, right-hand column	1,6-11, 27,29
P,X	BOUTONNIER ALAIN ET AL: "Preparation, immunogenicity, and protective efficacy, in a murine model, of a conjugate vaccine composed of the polysaccharide moiety of the lipopolysaccharide of Vibrio cholerae 0139 bound to tetanus toxoid." INFECTION AND IMMUNITY, vol. 69, no. 5, May 2001 (2001-05), pages 3488-3493, XP002209048 ISSN: 0019-9567 the whole document	1-29

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
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